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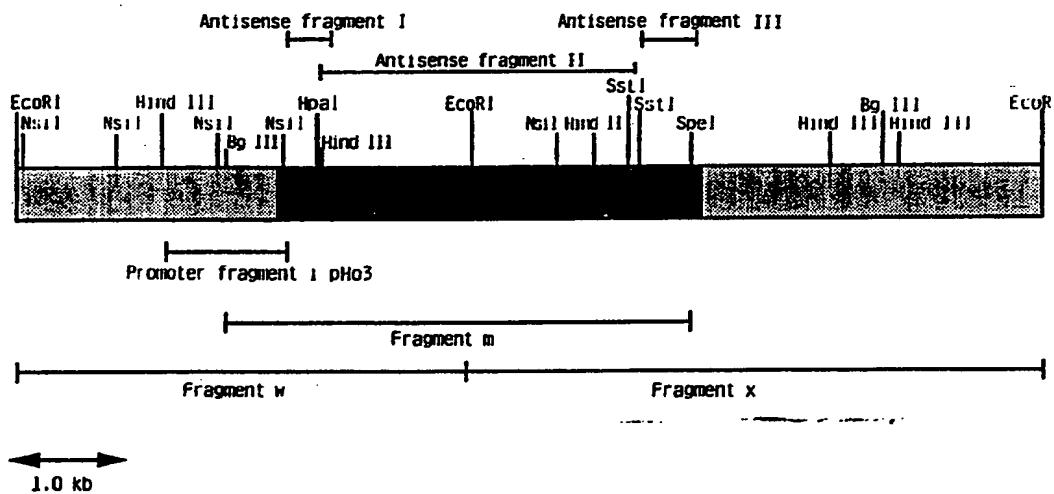
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Result of restriction analysis. GBSS coding region including introns are marked in a darker tone.



(57) Abstract

Genetically engineered modification of potato for suppressing the formation of amylose-type starch is described. Three fragments for insertion in the antisense direction into the potato genome are also described. Moreover, antisense constructs, genes and vectors comprising said antisense fragments are described. Further a promoter for the gene coding for formation of granule-bound starch synthase and also the gene itself are described. Also cells, plants, tubers, microtubers and seeds of potato comprising said antisense fragments are described. Finally, amylopectin-type starch, both native and derivatised, derived from the potato that is modified in a genetically engineered manner, as well as a method of suppressing amylose formation in potato are described.

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GENETICALLY ENGINEERED MODIFICATION OF POTATO TO
FORM AMYLOPECTIN-TYPE STARCH

The present invention relates to genetically engineered modification of potato, resulting in the formation of practically solely amylopectin-type starch in the potato. The genetically engineered modification implies the insertion of gene fragments into potato, said gene fragments comprising parts of leader sequence, translation 5 start, translation end and trailer sequence as well as coding and noncoding (i.e. exons and introns) parts of the gene for granule-bound starch synthase, inserted in 10 the antisense direction.

Background of the Invention

15 Starch in various forms is of great import in the food and paper industry. In future, starch will also be a great potential for producing polymers which are degradable in nature, e.g. for use as packing material. Many different starch products are known which are produced by 20 derivatisation of native starch originating from, inter alia, maize and potato. Starch from potato and maize, respectively, is competing in most market areas.

In the potato tuber, starch is the greatest part of the solid matter. About 1/4 to 1/5 of the starch in potato 25 is amylose, while the remainder of the starch is amylopectin. These two components of the starch have different fields of application, and therefore the possibility of producing either pure amylose or pure amylopectin is most interesting. The two starch components can be produced 30 from common starch, which requires a number of process steps and, consequently, is expensive and complicated.

It has now proved that by genetic engineering it is possible to modify potato so that the tubers merely produce mainly starch of one or the other type. As a result, 35 a starch quality is obtained which can compete in the areas where potato starch is normally not used today. Starch from such potato which is modified in a genetically

engineered manner has great potential as a food additive, since it has not been subjected to any chemical modification process.

Starch Synthesis

5 The synthesis of starch and the regulation thereof are presently being studied with great interest, both on the level of basic research and for industrial application. Although much is known about the assistance of certain enzymes in the transformation of saccharose 10 into starch, the biosynthesis of starch has not yet been elucidated. By making researches above all into maize, it has, however, been possible to elucidate part of the ways 15 of synthesis and the enzymes participating in these reactions. The most important starch-synthesising enzymes for producing the starch granules are the starch synthase and the branching enzyme. In maize, three forms of starch synthase have so far been demonstrated and studied, two of which are soluble and one is insolubly associated with the starch granules. Also the branching enzyme consists of 20 three forms which are probably coded by three different genes (Mac Donald & Preiss, 1985; Preiss, 1988).

The Waxy Gene in Maize

The synthesis of the starch component amylose essentially occurs by the action of the starch synthase alpha-25 -1,4-D-glucane-4-alpha-glucosyl transferase (EC 2.4.1.21) which is associated with the starch granules in the growth cell. The gene coding for this granule-bound enzyme is called "waxy" (= wx⁺), while the enzyme is called "GBSS" (granule-bound starch synthase).

30 waxy locus in maize has been thoroughly characterised both genetically and biochemically. The waxy gene on chromosome 9 controls the production of amylose in endosperm, pollen and the embryo sac. The starch formed in endosperm in normal maize with the wx⁺ allele consists to 25% of 35 amylose and to 75% of amylopectin. A mutant form of maize has been found in which the endosperm contains a mutation located to the wx⁺ gene, and therefore no functioning GBSS

is synthesised. Endosperm from this mutant maize therefore contains merely amylopectin as the starch component. This so-called waxy mutant thus contains neither GBSS nor amylose (Echt & Schwartz, 1981).

5 The GBSS protein is coded by the wx^+ gene in the cell nucleus but is transported to and active in the amyloplast. The preprotein therefore consists of two components, viz. a 7 kD transit peptide which transfers the protein across the amyloplast membrane, and the actual 10 protein which is 58 kD. The coding region of the wx^+ gene in maize is 3.7 kb long and comprises 14 exons and 13 introns. A number of the regulation signals in the promoter region are known, and two different polyadenylating sequences have been described (Klösgen et al, 1986; 15 Schwartz-Sommer et al, 1984; Shure et al, 1983).

Amylose Enzyme in Potato

In potato, a 60 kD protein has been identified, which constitutes the main granule-bound protein. Since antibodies against this potato enzyme cross-react with GBSS from 20 maize, it is assumed that it is the granule-bound synthase (Vos-Scheperkeuter et al, 1986). The gene for potato GBSS has, however, so far not been characterised to the same extent as the waxy gene in maize, either in respect of locating or structure.

25 Naturally occurring waxy mutants have been described for barley, rice and sorghum besides maize. In potato no natural mutant has been found, but a mutant has been produced by X-radiation of leaves from a monohaploid ($n=12$) plant (Visser et al, 1987). Starch isolated from tubers of 30 this mutant contains neither the GBSS protein nor amylose. The mutant is conditioned by a simple recessive gene and is called amf. It may be compared to waxy mutants of other plant species since both the GBSS protein and amylose are lacking. The stability of the chromosome number, however, 35 is weakened since this is quadrupled to the natural number ($n=48$), which can give negative effects on the potato plants (Jacobsen et al, 1990).

Inhibition of Amylose Production

The synthesis of amylose can be drastically reduced by inhibition of the granule-bound starch synthase, GBSS, which catalyses the formation of amylose. This inhibition 5 results in the starch mainly being amylopectin.

Inhibition of the formation of enzyme can be accomplished in several ways, e.g. by:

- mutagen treatment which results in a modification of the gene sequence coding for the formation of the enzyme
- 10 - incorporation of a transposon in the gene sequence coding for the enzyme
- genetically engineered modification so that the gene coding for the enzyme is not expressed, e.g. antisense gene inhibition.

15 Fig. 1 illustrates a specific suppression of normal gene expression in that a complementary antisense nucleotide is allowed to hybridise with mRNA for a target gene. The antisense nucleotide thus is antisense RNA which is transcribed in vivo from a "reversed" gene sequence
20 (Izant, 1989).

By using the antisense technique, various gene functions in plants have been inhibited. The antisense construct for chalcone synthase, polygalacturonase and phosphinotricin acetyltransferase has been used to inhibit the 25 corresponding enzyme in the plant species petunia, tomato and tobacco.

Inhibition of Amylose in Potato

In potato, experiments have previously been made to inhibit the synthesis of the granule-bound starch synthase 30 (GBSS protein) with an antisense construct corresponding to the gene coding for GBSS (this gene is hereinafter called the "GBSS gene"). Hergersberger (1988) describes a method by which a cDNA clone for the GBSS gene in potato has been isolated by means of a cDNA clone for the wx^+ 35 gene in maize. An antisense construct based on the entire cDNA clone was transferred to leaf discs of potato by means of *Agrobacterium tumefaciens*. In microtubers induced

in vitro from regenerated potato sprouts, a varying and very weak reduction of the amylose content was observed and shown in a diagram. A complete characterisation of the GBSS gene is not provided.

5 The gene for the GBSS protein in potato has been further characterised in that a genomic wx^+ clone was examined by restriction analysis. However, the DNA sequence of the clone has not been determined (Visser et al, 1989).

Further experiments with an antisense construct 10 responding to the GBSS gene in potato have been reported. The antisense construct which is based on a cDNA clone together with the CaMV 35S promoter has been transformed by means of Agrobacterium rhizogenes. According to information, the transformation resulted in a lower amylose 15 content in the potato, but no values have been accounted for (Flavell, 1990).

None of the methods used so far for genetically engineered modification of potato has resulted in potato with practically no amylose-type starch.

20 The object of the invention therefore is to provide a practically complete suppression of the formation of amylose in potato tubers.

Summary of the Invention

According to the invention, the function of the GBSS 25 gene and, thus, the amylose production in potato are inhibited by using completely new antisense constructs. For forming the antisense fragments according to the invention, the genomic GBSS gene is used as a basis in order to achieve an inhibition of GBSS and, consequently, of the 30 amylose production, which is as effective as possible. The antisense constructs according to the invention comprise both coding and noncoding parts of the GBSS gene which correspond to sequences in the region comprising promoter as well as leader sequence, translation start, translation 35 end and trailer sequence in the antisense direction. For a tissue-specific expression, i.e. the amylose production should be inhibited in the potato tubers only, use is made

of promoters which are specifically active in the potato tuber. As a result, the starch composition in other parts of the plant is not affected, which otherwise would give negative side-effects.

5 The invention thus comprises a fragment which essentially has one of the nucleotide sequences stated in SEQ ID No. 1, SEQ ID No. 2 or SEQ ID No. 3. However, the sequences may deviate from those stated by one or more non-adjacent base pairs, without affecting the function of
10 the fragments.

15 The invention also comprises a potato-tuber-specific promoter comprising 987 bp which belongs to the gene according to the invention, which codes for granule-bound starch synthase. Neither the promoter nor the corresponding gene has previously been characterised. The promoter sequence of 987 bp is stated in SEQ ID No. 4, while the gene sequence is stated in SEQ ID No. 5. Also the promoter and gene sequences may deviate from those stated by one or more non-adjacent base pairs, without affecting their
20 function.

25 The invention also comprises vectors including the antisense fragments and the antisense constructs according to the invention.

30 In other aspects the invention comprises cells, plants, tubers, microtubers and seeds whose genome contains the fragments according to the invention inserted in the antisense direction.

In still further aspects, the invention comprises amylopectin-type starch, both native and derivatised.

35 Finally, the invention comprises a method of suppressing amylose formation in potato, whereby mainly amylopectin-type starch is formed in the potato.

The invention will now be described in more detail with reference to the accompanying Figures in which

35 Fig. 1 illustrates the principle of the antisense gene inhibition,

Fig. 2 shows the result of restriction analysis of the potato GBSS gene,

Fig. 3 shows two new binary vectors pHo3 and pHo4,

Fig. 4 shows the antisense constructs pHoxwA, pHoxwB 5 and pHoxwD,

Fig. 5 shows the antisense constructs pHoxwF and pHoxwG, and

Fig. 6 shows the antisense constructs pHoxwK and pHoxwL.

10 Moreover, the sequences of the different DNA fragments according to the invention are shown in SEQ ID Nos 1, 2, 3, 4 and 5. There may be deviations from these sequences in one or more non-adjacent base pairs.

MATERIALS

15 In the practical carrying out of the invention the following materials were used:

Bacterial strains: E. coli DH5alpha and DH5alphaF'IQ(BRL). E. coli JM105 (Pharmacia). A. tumefaciens LBA4404 (Clontech).

20 Vectors: M13mp18 and mp19 (Pharmacia). pBI101 and pBI121 (Clontech). pBI240.7 (M. W. Bevan). pUC plasmids (Pharmacia).

25 Enzymes: Restriction enzymes and EcoRI linker (BRL). UNIONTM DNA Ligation Kit (Clontech). SequenaseTM DNA Sequencing Kit (USB). T₄-DNA ligase (Pharmacia).

The above-mentioned materials are used according to specifications stated by the manufacturers.

Genomic Library

30 A genomic library in EMBL3 has been produced by Clontech on the applicant's account, while using leaves of the potato Bintje as starting material.

Identification and Isolation of the GBSS Gene

35 The genomic library has been screened for the potato GBSS gene by means of cDNA clones for both the 5' and 3' end of the gene (said cDNA clones being obtained from M Hergersberger, Max Plank Institute in Cologne) according to a protocol from Clontech.

A full-length clone of the potato GBSS gene, wx311, has been identified and isolated from the genomic library. The start of the GBSS gene has been determined at an EcoRI fragment which is called fragment w (3.95 kb). The end of 5 the GBSS gene has also been determined at an EcoRI fragment which is called fragment x (5.0 kb). A BgIII-SpeI fragment which is called fragment m (3.9 kb) has also been isolated and shares sequences both from fragment w and from fragment x. The fragments w, m and x have been sub- 10 cloned in pUC13 (Viera, 1982; Yanisch-Peron et al, 1985) and are called pSw, pSm and pSx, respectively (Fig. 2).

Characterisation of the GBSS Gene in Potato

The GBSS gene in potato has been characterised by restriction analysis and cDNA probes, where the 5' and 3' 15 end of the GBSS gene has been determined more accurately (Fig. 2). Sequence determination according to Sanger et al, 1977 of the GBSS gene has been made on subclones from pSw and pSx in M13mp18 and mp19 as well as pUC19 starting around the 5' end (see SEQ ID No. 5).

20 The promoter region has been determined at a BgIII-NsII fragment (see SEQ ID No. 4). Transcription and translation start has been determined at an overlapping BgIII-HindIII fragment. The terminator region has in turn been determined at a SpeI-HindIII fragment.

25 Antisense Constructs for the GBSS Gene in Potato

The GBSS gene fragments according to the invention (see SEQ ID Nos 1, 2 and 3, and Fig. 2) have been determined in the following manner.

30 The restriction of pSw with NsII and HindIII gives fragment I (SEQ ID No. 1) which subcloned in pUC19 is called 19NH35. Further restriction of 19 NH35 with HpaI-SstI gives a fragment containing 342 bp of the GBSS gene according to the invention. This fragment comprises leader sequence, translation start and the first 125 bp of the 35 coding region.

The restriction of pSm with HpaI and NsiI gives fragment II (SEQ ID No. 2) which subcloned in pJRD184 (Heusterspreute et al, 1987) is called pJRDmitt. Further restriction of pJRDmitt with HpaI-SstI gives a fragment 5 containing 2549 bp of the GBSS gene according to the invention. This fragment comprises exons and introns from the middle of the gene.

The restriction of pSx with SstI and SpeI gives fragment III (SEQ ID No. 3) which subcloned in pBluescript 10 (Melton et al, 1984) is called pBlue3'. Further restriction of pBlue3' with BamHI-SstI gives a fragment containing 492 bp of the GBSS gene according to the invention. This fragment comprises the last intron and exon, translation end and 278 bp of trailer sequence.

15 Antisense Constructs with Fragment I (Fig. 4): For the antisense construct pHoxwA, the HpaI-SstI fragment from 19NH35 has been inserted in the antisense direction into the binary vector pBI121 (Jefferson et al, 1987) cleaved with SmaI-SstI. The transcription of the antisense fragment 20 is then initiated by the CaMV 35S promoter and is terminated by the NOS terminator (NOS = nopaline synthase).

For the antisense construct pHoxwB, the HpaI-SstI fragment from 19NH35 has been inserted in the antisense 25 direction into the binary vector pHo4 (Fig. 3) cleaved with SmaI-SstI. The patatin I promoter which is tuber specific in potato comes from the vector pBI240.7 obtained from M. Bevan, Institute of Plant Science Research, Norwich. The transcription of the antisense fragment is 30 then initiated by the patatin I promoter and is terminated by the NOS terminator.

For the antisense construct pHoxwD, the HpaI-SstI fragment from 19NH35 has been inserted in the antisense direction into the binary vector pHo3 (Fig. 3) cleaved 35 with SmaI-SstI. pHo3 is a new binary vector which is constructed on the basis of pBI101. This vector which contains the promoter according to the invention (see SEQ ID

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No. 4) (GBSS promoter) of the now characterised potato GBSS gene according to the invention has been restriction-cleaved with SmaI and SstI, the HpaI-SstI fragment from 19NH35 being inserted in the antisense direction. The 5 transcription of the antisense fragment is then initiated by its own GBSS promoter and is terminated by the NOS terminator. This means that the antisense fragment is transcribed only in the potato tuber, since the GBSS promoter like the patatin I promoter is tuber-specific.

10 Antisense Constructs with Fragment II (Fig. 5): For the antisense construct pHoxwF, the HpaI-SstI fragment from pJRDmitt has been inserted in the antisense direction into the binary vector pHo4 cleaved with SmaI-SstI. The transcription of the antisense fragment is then initiated by 15 the patatin I promoter and terminated by the NOS terminator.

For the antisense construct pHoxwG, the HpaI-SstI fragment from pJRDmitt has been inserted in the antisense direction into the binary vector pHo3 cleaved with SmaI-SstI. The transcription of the antisense fragment is then initiated by its own GBSS promoter and is terminated by 20 the NOS terminator.

25 Antisense Constructs with Fragment III (Fig. 6): For the antisense construct pHoxwK, the BamHI-SstI fragment from pBlue3' has been inserted in the antisense direction into the binary vector pHo4 cleaved with BamHI-SstI. The transcription of the antisense fragment is then initiated by the patatin I promoter and is terminated by the NOS terminator.

30 For the antisense construct pHoxwL, the BamHI-SstI fragment from pBlue3' has been inserted in the antisense direction into the binary vector pHo3 cleaved with BamHI-SstI. The transcription of the antisense fragment is then initiated by its own GBSS promoter and is terminated by 35 the NOS terminator.

The formed antisense constructs (Figs 4, 5, 6) have been transformed to *Agrobacterium tumefaciens* strain LBA4404 by direct transformation with the "freeze-thawing" method (Hoekema et al, 1983; An et al, 1988).

5 Transformation

The antisense constructs are transferred to bacteria, suitably by the "freeze-thawing" method (An et al, 1988). The transfer of the recombinant bacterium to potato tissue occurs by incubation of the potato tissue with the recombinant bacterium in a suitable medium after some sort of damage has been inflicted upon the potato tissue. During the incubation, T-DNA from the bacterium enters the DNA of the host plant. After the incubation, the bacteria are killed and the potato tissue is transferred to a solid medium for callus induction and is incubated for growth of callus.

After passing through further suitable media, sprouts are formed which are cut away from the potato tissue.

Checks for testing the expression of the antisense constructs and the transfer thereof to the potato genome are carried out by e.g. southern and northern hybridisation (Maniatis et al (1982)). The number of copies of the antisense construct which has been transferred is determined by southern hybridisation.

The testing of the expression on protein level is suitably carried out on microtubers induced in vitro on the transformed sprouts, thus permitting the testing to be performed as quickly as possible.

Characterisation of the GBSS Protein

The effect of the antisense constructs on the function of the GBSS gene with respect to the activity of the GBSS protein is examined by extracting starch from the microtubers and analysing it regarding the presence of the GBSS protein. In electrophoresis on polyacrylamide gel (Hovenkamp-Hermelink et al, 1987), the GBSS protein forms a distinct band at 60 kD, when the GBSS gene functions. When the GBSS gene is not expressed, i.e. when the anti-

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sense GBSS gene is fully expressed, thereby inhibiting the formation of GBSS protein, no 60 kD band is demonstrated on the gel.

Characterisation of the Starch

5 The composition of the starch in microtubers is identical with that of ordinary potato tubers, and therefore the effect of the antisense constructs on the amylose production is examined in microtubers. The proportion of amylose to amylopectin can be determined by a spectrophotometric method (e.g. according to Hovenkamp-Hermelink et al., 1988).

10 Extraction of Amylopectin from Amylopectin Potato
Amylopectin is extracted from the so-called amylopectin potato (potato in which the formation of amylose 15 has been suppressed by inserting the antisense constructs according to the invention) in a known manner.

15 Derivatisation of Amylopectin
Depending on the final use of the amylopectin, its physical and chemical qualities can be modified by derivatization. By derivatization is here meant chemical, physical and enzymatic treatment and combinations thereof (modified starches).

20 The chemical derivatization, i.e. chemical modification of the amylopectin, can be carried out in different ways, for example by oxidation, acid hydrolysis, dextrinisation, different forms of etherification, such as cationisation, hydroxy propylation and hydroxy ethylation, different forms of esterification, for example by vinyl acetate, acetic anhydride, or by monophosphatizing, 25 diphosphatizing and octenyl succination, and combinations thereof.

30 Physical modification of the amylopectin can be effected by e.g. cylinder-drying or extrusion.
In enzymatic derivatization, degradation (reduction 35 of the viscosity) and chemical modification of the amylopectin are effected by means of existing enzymatic systems.

The derivatisation is effected at different temperatures, according to the desired end product. The ordinary range of temperature which is used is 20-45°C, but temperatures up to 180°C are possible.

5 The invention will be described in more detail in the following Examples.

Example 1

Production of microtubers with inserted antisense constructs according to the invention

10 The antisense constructs (see Figs 4, 5 and 6) are transferred to *Agrobacterium tumefaciens* LBA 4404 by the "freeze-thawing" method (An et al, 1988). The transfer to potato tissue is carried out according to a modified protocol from Rocha-Sosa et al (1989).

15 Leaf discs from potato plants cultured in vitro are incubated in darkness on a liquid MS-medium (Murashige & Skoog; 1962) with 3% saccharose and 0.5% MES together with 100 µl of a suspension of recombinant *Agrobacterium* per 10 ml medium for two days. After these two days the bacteria are killed. The leaf discs are transferred to a solid medium for callus induction and incubated for 4-6 weeks, depending on the growth of callus. The solid medium is composed as follows:

MS + 3% saccharose

25 2 mg/l zeatin riboside
0.02 mg/l "NAA"
0.02 mg/l "GA₃"
500 mg/l "Claforan"
50 mg/l kanamycin
30 0.25% "Gellan"

Subsequently the leaf discs are transferred to a medium having a different composition of hormones, comprising:

MS + 3% saccharose
5 mg/l "NAA"
0.1 mg/l "BAP"
500 mg/l "Claforan"
5 50 mg/l kanamycin
0.25% "Gellan"

The leaf discs are stored on this medium for about 4 weeks, whereupon they are transferred to a medium in which the "Claforan" concentration has been reduced to 10 250 mg/l. If required, the leaf discs are then moved to a fresh medium every 4 or 5 weeks. After the formation of sprouts, these are cut away from the leaf discs and transferred to an identical medium.

The condition that the antisense construct has been 15 transferred to the leaf discs is first checked by analysing leaf extracts from the regenerated sprouts in respect of glucuronidase activity by means of the substrates described by Jefferson et al (1987). The activity is demonstrated by visual assessment.

20 Further tests of the expression of the antisense constructs and the transfer thereof to the potato genome are carried out by southern and northern hybridisation according to Maniatis et al (1981). The number of copies of the antisense constructs that has been transferred is determined by southern hybridisation.

When it has been established that the antisense constructs have been transferred to and expressed in the 30 potato genome, the testing of the expression on protein level begins. The testing is carried out on microtubers which have been induced in vitro on the transformed sprouts, thereby avoiding the necessity of waiting for the development of a complete potato plant with potato tubers.

35 Stem pieces of the potato sprouts are cut off at the nodes and placed on a modified MS medium. There they form microtubers after 2-3 weeks in incubation in darkness at 19°C (Bourque et al, 1987). The medium is composed as follows:

MS + 6% saccharose

2.5 mg/l kinetin

2.5 mg/l "Gellan"

The effect of the antisense constructs on the function of the GBSS gene in respect of the activity of the GBSS protein is analysed by means of electrophoresis on polyacrylamide gel (Hovenkamp-Hermelink et al, 1987). Starch is extracted from the microtubers and analysed regarding the presence of the GBSS protein. In a polyacrylamide gel, the GBSS protein forms a distinct band at 60 kD, when the GBSS gene functions. If the GBSS gene is not expressed, i.e. when the antisense GBSS gene is fully expressed so that the formation of GBSS protein is inhibited, no 60 kD band can be seen on the gel.

15 The composition of the starch, i.e. the proportion of amylose to amylopectin, is determined by a spectrophotometric method according to Hovenkamp-Hermelink et al (1988), the content of each starch component being determined on the basis of a standard graph.

20 Example 2

Extraction of amylopectin from amylopectin potato.

Potato whose main starch component is amylopectin, below called amylopectin potato, modified in a genetically engineered manner according to the invention, is grated, 25 thereby releasing the starch from the cell walls.

The cell walls (fibres) are separated from fruit juice and starch in centrifugal screens (centrisiler). The fruit juice is separated from the starch in two steps, viz. first in hydrocyclones and subsequently in specially 30 designed band-type vacuum filters.

Then a finishing refining is carried out in hydrocyclones in which the remainder of the fruit juice and fibres are separated.

The product is dried in two steps, first by predrying 35 on a vacuum filter and subsequently by final drying in a hot-air current.

Example 3

Chemical derivatisation of amylopectin

Amylopectin is sludged in water to a concentration of 20-50%. The pH is adjusted to 10.0-12.0 and a quaternary ammonium compound is added in such a quantity that the end product obtains a degree of substitution of 0.004-0.2. The reaction temperature is set at 20-45°C. When the reaction is completed, the pH is adjusted to 4-8, whereupon the product is washed and dried. In this manner the 10 cationic starch derivative 2-hydroxy-3-trimethyl ammonium propyl ether is obtained.

Example 4

Chemical derivatisation of amylopectin

Amylopectin is sludged in water to a water content of 10-25% by weight. The pH is adjusted to 10.0-12.0, and a quaternary ammonium compound is added in such a quantity that the end product obtains a degree of substitution of 0.004-0.2. The reaction temperature is set at 20-45°C. When the reaction is completed, the pH is adjusted to 4-8. 20 The end product is 2-hydroxy-3-trimethyl ammonium propyl ether.

Example 5

Chemical derivatisation of amylopectin

Amylopectin is sludged in water to a concentration of 20-50% by weight. The pH is adjusted to 5.0-12.0, and sodium hypochlorite is added so that the end product obtains the desired viscosity. The reaction temperature is set at 20-45°C. When the reaction is completed, the pH is adjusted to 4-8, whereupon the end product is washed and 30 dried. In this manner, oxidised starch is obtained.

Example 6

Physical derivatisation of amylopectin

Amylopectin is sludged in water to a concentration of 20-50% by weight, whereupon the sludge is applied to 35 a heated cylinder where it is dried to a film.

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Example 7

Chemical and physical derivatisation of amylopectin

Amylopectin is treated according to the process described in one of Examples 3-5 for chemical modification and is then further treated according to Example 6 for physical derivatisation.

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References:

- Mac Donald, F. D. and Preiss, J., 1985, *Plant. Physiol.* 78:849-852
- Preiss, J., 1988, In *The Biochemistry of Plants 14 Carbohydrates*. Ed. J. Preiss, Academic Press; 181-254
- 5 - Echt, C. S. and Schwarz, D., 1981, *Genetics* 99:275-284
- Klösgen, R. B., Gierl, A., Schwarz-Sommer, Z. and Saedler, H., 1986, *Mol. Gen. Genet.* 203:237-244
- Schwarz-Sommer, Z., Gierl, A., Klösgen, R. B., Wienand, U., Peterson, P. A. and Saedler, H., 1984, *EMBO J.* 3(5):1021-1028
- Shure, M., Wessler, S. and Fedoroff, N., 1983, *Cell* 35:225-233
- Jacobsen, E., Kriggsheld, H. T., Hovenkamp-Hermelink, J. H. M., Ponstein, A. S., Witholt, B. and Feenstra, W., 1990, *Plant. Sci.* 67:177-182
- 15 - Visser, R. G. F., Hovenkamp-Hermelink, J. H. M., Ponstein, A. S., Vos-Scheperskeuter, G. H., Jacobsen, E., Feenstra, W. J. and Witholt, B., 1987, Proc. 4th European Congress on Biotechnology 1987, Vol. 2, Elsevier, Amsterdam; 432-435
- Vos-Scheperskeuter, G. H., De Boer, W., Visser, R. G. F., Feenstra, W. J. and Witholt, B., 1986, *Plant. Physiol.* 82:411-416
- 20 - Cornelissen, M., 1989, *Nucleic Acids Res.* 17(18):7203-7209
- Izant, J. G., 1989, *Cell Motility and Cytoskeleton* 14:81-91
- Sheehy, R. E., Kramer, M., Hiatt, W. R., 1988, Proc. Natl. Acad. Sci. USA, 85(23):8805-8809
- 25 - Van der Krol, A. R., Mur, L. A., de Lange, P., Gerats, A. G. M., Mol, J. N. M. and Stuitje, A. R., 1990, *Mol. Gen. Genet.* 220:204-212
- Flavell, R. B., 1990, *AgBiotech. News and Information* 2(5):629-630
- 30 - Hergersberger, M., 1988, *Molekulare Analyse des waxy Gens aus Solanum tuberosum und Expression von waxy*

antisense RNA in transgenen Kartoffeln. Thesis for a doctorate from the University in Cologne

- Visser, R. G. F., Hergersberger, M., van der Leij, F. R., Jacobsen, E., Witholt, B. and Feenstra, W. J., 1989,

5 Plant. Sci. 64:185-192

An, G., Ebert, P. R., Mitra, A. and Ha, S. B., 1987, Plant Mol. Biol. Manual A3:1-19

- Hoekema, A., Hirsch, P. R., Hooykaas, P. J. J. and Schilperoort, R. A., 1983, Nature 303:179-180

10 - Jefferson, R. A., Kavanagh, T. A. and Bevan, M. W., 1987, EMBO J. 6:3201-3207

- Sanger, F., Nicklen, S. and Coulson, A. R., 1977, Proc. Natl. Acad. Sci. USA 74:5463-5467

- Viera, J. and Messing, J., 1982, Gene 19:259-268

15 - Yanisch-Perron, C., Viera, J. and Messing, J., 1985, Gene 33:103-119

- Heusterspreute et al (1987) Gene 53:294-300

- Melton, D. A. et al (1984), Nucleic Acids Res. 12:7035-7056 (the plasmide is sold by Stratagene)

20 - Murashige, T. and Skoog, F., 1962, Physiol. Plant 15:473-497.

- Rocha-Sosa, M., Sonnewald, U., Frommer, W., Stratmann, M., Shell, J. and Willmitzer, L., 1989, EMBO J., 8(1):23-29

25 - Jefferson, R. A., Kavanagh, R. A. and Bevan, M. W., 1987, EMBO J. 6:3901-3907

- Maniatis, T., Fritsch, E. F. and Sambrook, J., 1982, Molecular Cloning, A Laboratory Handbook, Cold Spring Harbor Laboratory Press, Cold Spring Harbor

30 - Bourque, J. E., Miller, J. C. and Park, W. D., 1987, In Vitro Cellular & Development Biology 23(5):381-386

- Hovenkamp-Hermelink, J. H. M., Jacobsen, E., Ponstein, A. S., Visser, R. G. F., Vos-Scheperkeuter, G. H., Bijmolt, E. W., de Vries, J. N., Witholt, B. J. &

35 Feenstra, W. J., 1987, Theor. Appl. Genet. 75:217-221

- Hovenkamp-Hermelink, J. H. M., de Vries, J. N., Adamse, P., Jacobsen, E., Witholt, B. and Feenstra, W. J., 1988,

20

Potato Research 31:241-246

- Modified starches: Properties and use D. B. Wurzburg
- Bevan, M. W., 1984. Nucleic Acids Res. 12:8711-8721.

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SEQ ID No. 1

Sequenced molecule: genomic DNA

Name: GBSS gene fragment from potato

Length of sequence: 342 bp

TGCATGTTTC	CCTACATTC	ATTTAGAAC	TGTTGTTGGT	GTATAAACGT	50
TGTTTCA	ATATCTCAT	CTATTCTGAT	TTGATTCTC	TTGCCTACTG	100
TAATCGGTGA	TAAATGTGAA	TGCTTCCTT	CTTCTCAGAA	ATCAATTCT	150
GT	TTTGTGTTT	TGTTCATCTG	TAGCTTATTC	TCTGGTAGAT	200
GTAGACCACA	CATCAC	ATG GCA AGC	ATC ACA GCT	TCA CAC CAC	243
		Met Ala Ser Ile	Thr Ala Ser	His His	
		1	5		
TTT	GTG TCA AGA AGC	CAA ACT TCA CTA	GAC ACC AAA	TCA ACC	285
Phe	Val Ser Arg Ser	Gln Thr Ser Leu	Asp Thr Lys	Ser Thr	
10	15	20			
TTG	TCA CAG ATA GGA	CTC AGG AAC CAT	ACT CTG ACT	CAC AAT	327
Leu	Ser Gln Ile Gly	Leu Arg Asn	His Thr Leu	Thr His Asn	
25	30	35			
GGT	TTA AGG GCT	GTT			342
Gly	Leu Arg Ala Val				
	40				

22

SEQ ID No. 2

Sequenced molecule: genomic DNA

Name: GBSS gene fragment from potato

Length of sequence: 2549 bp

AAC AAG CTT GAT GGG CTC CAA TCA ACA ACT AAT ACT AAG GTA	42
Asn Lys Leu Asp Gly Leu Gln Ser Thr Thr Asn Thr Lys Val	
45 50 55	
ACA CCC AAG ATG GCA TCC AGA ACT GAG ACC AAG AGA CCT GGA	84
Thr Pro Lys Met Ala Ser Arg Thr Glu Thr Lys Arg Pro Gly	
60 65 70	
TGC TCA GCT ACC ATT GTT TGT GGA AAG GGA ATG AAC TTG ATC	126
Cys Ser Ala Thr Ile Val Cys Gly Lys Gly Met Asn Leu Ile	
75 80	
TTT GTG GGT ACT GAG GTT GGT CCT TGG AGC AAA ACT GGT GGA	168
Phe Val Gly Thr Glu Val Gly Pro Trp Ser Lys Thr Gly Gly	
85 90 95	
CTA GGT GAT GTT CTT GGT GGA CTA CCA CCA GCC CTT GCA	207
Leu Gly Asp Val Leu Gly Gly Leu Pro Pro Ala Leu Ala	
100 105 110	
GTAAGTCITT CTTTCATTG GTTACCTACT CATTCAATTAC TTATTTGTT	257
TAGTTAGTTT CTACTGCATC AGTCTTTTA TCATTTAG GCC CGC GGA	304
Ala Arg Gly	
CAT CGG GAA ATG ACA ATA TCC CCC CGT TAT GAC CAA TAC AAA	346
His Arg Val Met Thr Ile Ser Pro Arg Tyr Asp Gln Tyr Lys	
115 120 125	
GAT GCT TGG GAT ACT GGC GTT GCG GTT GAG	386
Asp Ala Trp Asp Thr Gly Val Ala Val Glu	
130 135	
GTACATCTTC	
CTATATTGAT ACGGTACAAT ATTGTTCTCT TACATTCCT GATTCAAGAA	436
TGTGATCATC TGCAG GTC AAA GTT GGA GAC AGC ATT GAA ATT GTT	481
Val Lys Val Gly Asp Ser Ile Glu Ile Val	
140 145	
CGT TTC TTT CAC TGC TAT AAA CGT GGG GTT GAT CGT GTT TTT	523
Arg Phe Phe His Cys Tyr Lys Arg Gly Val Asp Arg Val Phe	
150 155 160	
GTT GAC CAC CCA ATG TTC TGG GAG AAA	560
Val Asp His Pro Met Phe Leu Glu Lys	
165 170	

23

TATGATTATG AATCCGTCTT GAGGGATACG CAGAACAGGT CATTTGAGT	610
ATCTTTAAC TCTACTGGTG CTTTTACTCT TTTAAG GTT TGG GGC AAA	658
Val Trp Gly Lys	
175	
ACT GGT TCA AAA ATC TAT GGC CCC AAA GCT GGA CTA GAT TAT	700
Thr Gly Ser Lys Ile Tyr Gly Pro Lys Ala Gly Leu Asp Tyr	
180	185
CTG GAC AAT GAA CTT AGG TTC AGC TTG TTG TGT CAA	736
Leu Asp Asn Glu Leu Arg Phe Ser Leu Leu Cys Gln	
190	195
200	
GTAAGTTAGT TACTCTTGAT TTTTATGTGG CATTTCAGTC TTTTGTCTTT	786
AATCGTTTT TTAACCTTGT TTTCTCAG GCA GCC CTA GAG GCA CCT	832
Ala Ala Leu Glu Ala Pro	
205	
AAA GTT TTG AAT TTG AAC AGT AGC AAC TAC TTC TCA GGA CCA	874
Lys Val Leu Asn Leu Asn Ser Ser Asn Tyr Phe Ser Gly Pro	
210	215
220	
TAT G GTAATTAACA CATCCTAGTT TCAGAAAACT CCTTACTATA	918
Tyr G	
TCATTGTAGG TAATCATCTT TATTTGCCT ATTCCCTGCAG GA GAG GAT	966
ly Glu Asp	
225	
GTT CTC TTC ATT GCC AAT GAT TGG CAC ACA GCT CTC ATT CCT	1008
Val Leu Phe Ile Ala Asn Asp Trp His Thr Ala Leu Ile Pro	
230	235
TGC TAC TTG AAG TCA ATG TAC CAG TCC AGA GGA ATC TAC TTG	1050
Cys Tyr Leu Lys Ser Met Tyr Gln Ser Arg Gly Ile Tyr Leu	
240	245
250	
AAT GCC AAG GTAAAATTTC TTTGTATTCA CTCGATTGCA	1089
Asn Ala Lys	
255	
CGTTACCCCTG CAAATCAGTA AGGTTGTATT AATATATGAT AAATTCACAA	1139
TTGCCTCCAG GTT GCT TTC TGC ATC CAT AAC ATT GCC TAC CAA	1182
Val Ala Phe Cys Ile His Asn Ile Ala Tyr Gln	
260	265
GGT CGA TTT TCT TTC TCT GAC TTC CCT CTT CTC AAT CTT CCT	1224
Gly Arg Phe Ser Phe Ser Asp Phe Pro Leu Leu Asn Leu Pro	
270	275
280	
GAT GAA TTC AGG GGT CCT TTT GAT TTC ATT GAT GGG TAT	1263
Asp Glu Phe Arg Gly Ser Phe Asp Phe Ile Asp Gly Tyr	
285	290
GTATTTATGG TTGAAATCAG ACCTCCAACT TTTGAGCTG TTTGGATGCT	1313

24

AGTAAATTGA GTTTTAAAAA TTTGCAGAT ATGAG AAG CCT GTT AAG Lys Pro Val Lys 295	1360
GGT AGG AAA ATC AAC TGG ATG AAG GCT GGG ATA TTA GAA TCA Gly Arg Lys Ile Asn Trp Met Lys Ala Gly Ile Leu Glu Ser 300 305 310	1402
CAT AGG GTG GTT ACA GTG AGC CCA TAC TAT GCC CAA GAA CTT His Arg Val Val Thr Val Ser Pro Tyr Tyr Ala Gln Glu Leu 315 320 325	1444
GTC TCT GCT GTT GAC AAG GGA GTT GAA TTG GAC AGT GTC CTT Val Ser Ala Val Asp Lys Gly Val Glu Leu Asp Ser Val Leu 330 335 340	1486
CGT AAG ACT TGC ATA ACT GGG ATT GTG AAT GGC ATG GAT ACA Arg Lys Thr Cys Ile Thr Gly Ile Val Asn Gly Met Asp Thr 345 350	1528
CAA GAG TGG AAC CCA GCG ACT GAC AAA TAC ACA GAT GTC AAA Gln Glu Trp Asn Pro Ala Thr Asp Lys Tyr Thr Asp Val Lys 355 360 365	1570
TAC GAT ATA ACC ACT GTAAAGATAAG ATTTTCCGA CTCCAGTATA Tyr Asp Ile Thr Thr 370	1615
TACTAAATTAA TTTGTATGT TTATGAAATT AAAGAGTTCT TGCTAATCAA AATCTCTATA CAG GTC ATG GAC GCA AAA CCT TTA CTA AAG GAG Val Met Asp Ala Lys Pro Leu Leu Lys Glu 375 380	1665 1708
GCT CTT CAA GCA GCA GTT GGC TTG CCT GTT GAC AAG AAG ATC Ala Leu Gln Ala Ala Val Gly Leu Pro Val Asp Lys Lys Ile 385 390 395	1756
CCT TTG ATT GGC TTC ATC GGC AGA CTT GAG GAG CAG AAA GGT Pro Leu Ile Gly Phe Ile Gly Arg Leu Glu Glu Gln Lys Gly 400 405 410	1792
TCA GAT ATT CTT GTT GCT GCA ATT CAC AAG TTC ATC GGA TTG Ser Asp Ile Leu Ala Val Ala Ile His Lys Phe Ile Gly Leu 415 420 425	1834
GAT GTT CAA ATT GTA GTC CTT GTAAAGTACCA AATGGACTCA Asp Val Gln Ile Val Val Leu 430	1875
TGGTATCTCT CTTGTTGAGT TTACTTGTGC CGAAACTGAA ATGGACCTGC TACTCATCTT ATGCAATGAG GGA ACT GGC AAA ARG GAG TTT GAG Gly Thr Gly Lys Lys Glu Phe Glu 435 440	1925 1968

25

CAG GAG ATT GAA CAG CTC GAA GTG TTG TAC CCT AAC AAA GCT Gln Glu Ile Glu Gln Leu Glu Val Leu Tyr Pro Asn Lys Ala 445 450	2010
AAA GGA GTG GCA AAA TTC AAT GTC CCT TTG GCT CAC ATG ATC Lys Gly Val Ala Lys Phe Asn Val Pro Leu Ala His Met Ile 455 460 465	2052
ACT GCT GGT GCT GAT TTT ATG TTG GTT CCA AGC AGA TTT GAA Thr Ala Gly Ala Asp Phe Met Leu Val Pro Ser Arg Phe Glu 470 475 480	2094
CCT TGT GGT CTC ATT CAG TTA CAT GCT ATG CGA TAT GGA ACA Pro Cys Gly Leu Ile Gln Leu His Ala Met Arg Tyr Gly Thr 485 490 495	2136
GTAAGAACCA GAAGAGCTTG TACCTTTTA CTGAGTTTTT AAAAAAGAA TCATAAGACC TTGTTTCCA TCTAAAGTTT AATAACCAAC TAAATGTTAC TGCAGCAAGC TTTTCATTTC TGAAAATTGG TTATCTGATT TTAACGTAAT CACATGTGAG TCAG GTA CCA ATC TGT GCA TCG ACT GGT GGA CTT Val Pro Ile Cys Ala Ser Thr Gly Gly Leu 500 505	2186 2236 2286 2330
GTT GAC ACT GTG AAA GAA GGC TAT ACT GGA TTC CAT ATG GGA Val Asp Thr Val Lys Glu Gly Tyr Thr Gly Phe His Met Gly 510 515 520	2372
GCC TTC AAT GTT GAA GTATGTGATT TTACATCAAT TGTGTACTTG Ala Phe Asn Val Glu 525	2417
TACATGGTCC ATTCTCGTCT TGATATAACCC CTTGTTGCAT AAACATTAAC TTATTGCTTC TTGAATTTGG TTAG TGC GAT GTT GTT GAC CCA GCT Cys Asp Val Val Asp Pro Ala 530	2467 2512
GAT GTG CTT AAG ATA GTA ACA ACA GTT GCT AGA GCT C Asp Val Leu Lys Ile Val Thr Thr Val Ala Arg Ala 535 540	2549

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SEQ ID No. 3

Sequenced molecule: genomic DNA

Name: GBSS gene fragment from potato

Length of sequence: 492 bp

GAG CTC TCC TGG AAG	GTAAGTGTGA ATTTGATAAT TTGCGTAGGT	45
Glu Leu Ser Trp Lys		
565		
ACTTCAGTTT GTTGTCTCG TCAGCACTGA TGGATTCCAA CTGGTGTCT		95
TGCAG GAA CCT GCC AAG AAA TGG GAG ACA TTG		127
Glu Pro Ala Lys Lys Trp Glu Thr Leu		
570 575		
CTA TTG GGC TTA GGA GCT TCT GGC AGT GAA CCC GGT GTT GAA		169
Leu Leu Gly Leu Gly Ala Ser Gly Ser Glu Pro Gly Val Glu		
580 585 590		
GGG GAA GAA ATC GCT CCA CTT GCC AAG GAA AAT GTA GCC ACT		211
Gly Glu Glu Ile Ala Pro Leu Ala Lys Glu Asn Val Ala Thr		
595 600 605		
CCT TAA ATGAGCTTTG GTTATCCTTG TTTCAACAAT AAGATCATTA		257
Pro ***		
606		
AGCAAACGTA TTTACTAGCG AACTATGTAG AACCCCTATT A TGGGGTCTCA		307
ATCATCTACA AAATGATTGG TTTTGCTGG GGAGCAGCAG CATATAAGGC		357
TGTAAAATCC TGGTTAATGT TTTTAGGT AAGGGCTATT TAAGGTGGTG		407
TGGATCAAAG TCAATAGAAA ATAGTTATT CTAACGTTG CAACTAAATA		457
CTTAGTAAATG TAGCATAAAAT AATACTAGAA CTAGT		492

SEQ ID No. 4

Sequenced molecule: genomic DNA

Name: Promoter for the GBSS gene from potato

Length of sequence: 987 bp

AAGCTTTAAC	GAGATAGAAA	ATTATGTTAC	TCCGTTTGT	TCATTACTTA	50
ACAAATGCAA	CAGTATCTTG	TACCAAATCC	TTTCTCTCTT	TCAAACTTT	100
TCTATTGGC	TGTTGACGGA	GTAATCAGGA	TACAAACCAC	AAGTATTTAA	150
TTGACTCCTC	CGCCAGATAT	TATGATTAT	GAATCCTCGA	AAAGCCTATC	200
CATTAAGTCC	TCATCTATGG	ATATACTTGA	CAGTATCTTC	CTGTTGGGT	250
ATTTTTTTT	CCTGCCAAGT	GGAACGGAGA	CATGTTATGA	TGTATACGGG	300
AAGCTCGTTA	AAAAAAAATA	CAATAGGAAG	AAATGTAACA	AACATTGAAT	350
GTTGTTTTA	ACCATCCTTC	CTTAGCAGT	GTATCAATT	TGTAATAGAA	400
CCATGCATCT	CAATCTTAAT	ACTAAAATGC	AACTTAATAT	AGGCTAAACC	450
AAGATAAAGT	AATGTATTCA	ACCTTTAGAA	TTGTGCATTC	ATAATTAGAT	500
CTTGTGTTGTC	GTAAAAAAATT	AGAAAATATA	TTTACAGTAA	TTTGGAAATAC	550
AAAGCTAAGG	GGGAAGTAAC	TAATATTCTA	GTGGAGGGAG	GGACCAAGTAC	600
CAGTACCTAG	ATATTATTTT	TAATTACTAT	AATAATAATT	TAATTAACAC	650
GAGACATAGG	AATGTCAAGT	GGTAGCGTAG	GAGGGAGTTG	GTTTAGTTT	700
TTAGATACTA	GGAGACAGAA	CCGGACGGCC	CATTGCAAGG	CCAAGTTGAA	750
GTCCAGCCGT	GAATCAACAA	AGAGAGGGCC	CATAATACTG	TCGATGAGCA	800
TTTCCCTATA	ATACAGTGTC	CACAGTTGCC	TTCTGCTAAG	GGATAGCCAC	850
CCGCTATTCT	CTTGACACGT	GTCACTGAAA	CCTGCTACAA	ATAAGGCAGG	900
CACCTCCTCA	TTCTCACTCA	CTCACTCACA	CAGCTCAACA	AGTGGTAACT	950
TTTACTCAGTC	TCCTCCAATT	ATTCTGATT	TCATGCA		987

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SEQ ID No. 5

Sequenced molecule: genomic DNA

Name: GBSS gene from potato

Length of sequence: 4964 bp

AAGCTTTAAC	GAGATAGAAA	ATTATGTTAC	TCCGTTTGT	TCATTACTTA	50
ACAAATGCAA	CAGTATCTTG	TACCAAATCC	TTTCTCTCTT	TTCAAACCTT	100
TCTATTGGC	TGTTGACGGA	GTAATCAGGA	TACAAACCAC	AAGTATTAA	150
TTGACTCCTC	CGCCAGATAT	TATGATTAT	GAATCCTCGA	AAAGCCTATC	200
CATTAAGTCC	TCATCTATGG	ATATACTGGA	CAGTATCTTC	CTGTTGGGT	250
ATTTTTTTT	CCTGCCAAGT	GGAACGGAGA	CATGTTATGA	TGTATACGGG	300
AAGCTCGTTA	AAAAAAAATA	CAATAGGAAG	AAATGTAACA	AACATTGAAT	350
GTTGTTTTA	ACCATCCTTC	CTTAGCAGT	GTATCAATT	TGTAATAGAA	400
CCATGCATCT	CAATCTTAAT	ACTAAAATGC	AACTTAATAT	AGGCTAAACC	450
AAGATAAAGT	AATGTATTCA	ACCTTTAGAA	TTGTGCATT	ATAATTAGAT	500
CTTGTGTC	GTAAAAAATT	AGAAAATATA	TTTACAGTAA	TTTGGAAATAC	550
AAAGCTAAGG	GGGAAGTAAC	TAATATTCTA	GTGGAGGGAG	GGACCAGTAC	600
CAGTACCTAG	ATATTATTT	TAATTACTAT	ATAATAATT	TAATTAACAC	650
GAGACATAGG	AATGTCAAGT	GGTAGCGTAG	GAGGGAGTTG	GTTAGTTT	700
TTAGATACTA	GGAGACAGAA	CCGGACGGCC	CATTGCAAGG	CCAAGTTGAA	750
GTCCAGCCGT	GAATCAACAA	AGAGAGGGCC	CATAATACTG	TCGATGAGCA	800
TTTCCCTATA	ATACAGTGT	CACAGTTGCC	TTCTGCTAAG	GGATAGCCAC	850
CCGCTATTCT	CTTGACACGT	GTCACTGAAA	CCTGCTACAA	ATAAGGCAGG	900
CACCTCCTCA	TTCTCACTCA	CTCACTCACA	CAGCTCAACA	AGTGGTAAC	950
TTTACTCATC	TCCTCCAATT	ATTCTGATT	TCATGCATGT	TTCCCTACAT	1000
TCTATTATGA	ATCGTGGTGT	GGTGTATAAA	CGTTGTTCA	TATCTCATCT	1050
CATCTATTCT	GATTTGATT	CTCTTGCTA	CTGTAATCGG	TGATAAATGT	1100
GAATGCTTCC	TTTCTCTCA	GAAATCAATT	TCTGTTTGT	TTTGTTTCAT	1150
CTGTAGCTTA	TTCTCTGGTA	GATTCCCCTT	TTTGTAGACC	ACACATCAC	1199
ATG GCA AGC ATC ACA GCT TCA CAC CAC TTT GTG	TCA AGA AGC				1241
Met Ala Ser Ile Thr Ala Ser His His Phe Val Ser Arg Ser					
1	5	10			
CAA ACT TCA CTA GAC ACC AAA TCA ACC TTG TCA CAG ATA GGA					1283
Gln Thr Ser Leu Asp Thr Lys Ser Thr Leu Ser Gln Ile Gly					
15	20	25			
CTC AGG AAC CAT ACT CTG ACT CAC AAT GGT TTA AGG GCT GTT					1325
Leu Arg Asn His Thr Leu Thr His Asn Gly Leu Arg Ala Val					
30	35	40			
AAC AAG CTT GAT GGG CTC CAA TCA ACA ACT AAT ACT AAG GTA					1367
Asn Lys Leu Asp Gly Leu Gln Ser Thr Thr Asn Thr Lys Val					
45	50	55			
ACA CCC AAG ATG GCA TCC AGA ACT GAG ACC AAG AGA CCT GGA					1409
Thr Pro Lys Met Ala Ser Arg Thr Glu Thr Lys Arg Pro Gly					
60	65	70			
TGC TCA GCT ACC ATT GTT TGT GGA AAG GGA ATG AAC TTG ATC					1451
Cys Ser Ala Thr Ile Val Cys Gly Lys Gly Met Asn Leu Ile					
75	80				
TTT GTG GGT ACT GAG GTT GGT CCT TGG AGC AAA ACT GGT GGA					1493
Phe Val Gly Thr Glu Val Gly Pro Tyr Ser Lys Thr Gly Gly					
85	90	95			

CTA GGT GAT GTT CTT GGT GGA CTA CCA CCA GCC CTT GCA Leu Gly Asp Val Leu Gly Gly Leu Pro Pro Ala Leu Ala 100 105 110	1532
GTAAGTCTT CTTTCATTTG GTTACCTACT CATTCAATTAC TTATTTGTT TAGTTAGTT CTACTGCATC AGTCTTTA TCATTTAG GCC CGC GGA Ala Arg Gly	1582 1629
CAT CGG GTA ATG ACA ATA TCC CCC CGT TAT GAC CAA TAC AAA His Arg Val Met Thr Ile Ser Pro Arg Tyr Asp Gln Tyr Lys 115 120 125	1671
GAT GCT TGG GAT ACT GGC GTT GCG GTT GAG GTACATCTTC Asp Ala Trp Asp Thr Gly Val Ala Val Glu 130 135	1711
CTATATTGAT ACGGTACAAT ATTGTTCTCT TACATTCCT GATTCAAGAA TGTGATCATC TGCAG GTC AAA GTT GGA GAC AGC ATT GAA ATT GTT Val Lys Val Gly Asp Ser Ile Glu Ile Val 140 145	1761 1806
CGT TTC TTT CAC TGC TAT AAA CGT GGG GTT GAT CGT GTT TTT Arg Phe Phe His Cys Tyr Lys Arg Gly Val Asp Arg Val Phe 150 155 160	1848
GTT GAC CAC CCA ATG TTC TTG GAG AAA GTAAGCATAT Val Asp His Pro Met Phe Leu Glu Lys 165 170	1885
TATGATTATG AATCCGTCT GAGGGATACG CAGAACAGGT CATTGAGT ATCTTTAAC TCTACTGGTG CTTTACTCT TTTAAG GTT TGG GGC AAA Val Trp Gly Lys 175	1935 1983
ACT GGT TCA AAA ATC TAT GGC CCC AAA GCT GGA CTA GAT TAT Thr Gly Ser Lys Ile Tyr Gly Pro Lys Ala Gly Leu Asp Tyr 180 185	2025
CTG GAC AAT GAA CTT AGG TTC AGC TTG TTG TGT CAA Leu Asp Asn Glu Leu Arg Phe Ser Leu Leu Cys Gln 190 195 200	2061
GTAAGTTAGT TACTCTTGAT TTTTATGTGG CATTTCATCTC TTTTGTCTT AATCGTTTT TTAACCTTGT TTTCTCAG GCA GCC CTA GAG GCA CCT Ala Ala Leu Glu Ala Pro 205	2111 2157
AAA GTT TTG AAT TTG AAC AGT AGC AAC TAC TTC TCA GGA CCA Lys Val Leu Asn Leu Asn Ser Ser Asn Tyr Phe Ser Gly Pro 210 215 220	2199

30

TAT G	GTAATTAACA CATCCTAGTT TCAGAAA ACT CCTTACTATA	2243
Tyr G		
TCATTGTAGG TAATCATCTT TATTTGCCT ATTCCCTGCAG GA GAG GAT	2291	
ly Glu Asp		
225		
GTT CTC TTC ATT GCC AAT GAT TGG CAC ACA GCT CTC ATT CCT	2333	
Val Leu Phe Ile Ala Asn Asp Trp His Thr Ala Leu Ile Pro		
230	235	
TGC TAC TTG AAG TCA ATG TAC CAG TCC AGA GGA ATC TAC TTG	2375	
Cys Tyr Leu Lys Ser Met Tyr Gln Ser Arg Gly Ile Tyr Leu		
240	245 250	
AAT GCC AAG	GTAAAATTTC TTTGTATTCA CTCGATTGCA	2414
Asn Ala Lys		
255		
CGTTACCCG CAAATCAGTA AGGTTGTATT AATATATGAT AAATTCACA	2464	
TTGCCTCCAG GTT GCT TTC TGC ATC CAT AAC ATT GCC TAC CAA	2507	
Val Ala Phe Cys Ile His Asn Ile Ala Tyr Gln		
260	265	
GGT CGA TTT TCT TTC TCT GAC TTC CCT CTT CTC AAT CTT CCT	2549	
Gly Arg Phe Ser Phe Ser Asp Phe Pro Leu Leu Asn Leu Pro		
270	275 280	
GAT GAA TTC AGG GGT TCT TTT GAT TTC ATT GAT GGG TAT	2588	
Asp Glu Phe Arg Gly Ser Phe Asp Phe Ile Asp Gly Tyr		
285	290	
GTATTTATGC TTGAAATCAG ACCTCCA ACT TTTGAAGCTC TTTTGATGCT	2638	
AGTAAATTGA GTTTTTAAAAA TTTTGCAGAT ATGAG AAG CCT GTT AAG	2685	
Lys Pro Val Lys		
295		
GGT AGG AAA ATC AAC TGG ATG AAG GCT GGG ATA TTA GAA TCA	2727	
Gly Arg Iys Ile Asn Trp Met Lys Ala Gly Ile Leu Glu Ser		
300	305 310	
CAT AGG GTG GTT ACA GTG AGC CCA TAC TAT GCC CAA GAA CTT	2769	
His Arg Val Val Thr Val Ser Pro Tyr Tyr Ala Gln Glu Leu		
315	320 325	
GTC TCT GCT GTT GAC AAG GGA GTT GAA TTG GAC AGT GTC CTT	2811	
Val Ser Ala Val Asp Lys Gly Val Glu Leu Asp Ser Val Leu		
330	335 340	
CGT AAG ACT TGC ATA ACT GGG ATT GTG AAT GGC ATG GAT AGA	2853	
Arg Lys Thr Cys Ile Thr Gly Ile Val Asn Gly Met Asp Thr		
345	350	

31

CAA GAG TGG AAC CCA GCG ACT GAC AAA TAC ACA GAT GTC AAA Gln Glu Trp Asn Pro Ala Thr Asp Lys Tyr Thr Asp Val Lys 355	360	365	2895
TAC GAT ATA ACC ACT	GTAAGATAAG ATTTTTCCGA CTCCAGTATA		2940
Tyr Asp Ile Thr Thr 370			
TTCTAAATTA TTTTGTATGT TTATGAAATT AAAGAGTTCT TGCTAATCAA AATCTCTATA CAG GTC ATG GAC GCA AAA CCT TTA CTA AAG GAG Val Met Asp Ala Lys Pro Leu Leu Lys Glu 375	380		2990 3033
GCT CTT CAA GCA GCA GTT GGC TTG CCT GTT GAC AAG AAG ATC Ala Leu Gln Ala Ala Val Gly Leu Pro Val Asp Lys Lys Ile 385	390	395	3075
CCT TTG ATT GGC TTC ATC GGC AGA CTT GAG GAG CAG AAA GGT Pro Leu Ile Gly Phe Ile Gly Arg Leu Glu Glu Gln Lys Gly 400	405	410	3117
TCA GAT ATT CTT GTT GCT GCA ATT CAC AAG TTC ATC GGA TTG Ser Asp Ile Leu Ala Val Ala Ile His Lys Phe Ile Gly Leu 415	420	425	3159
GAT GTT CAA ATT GTA GTC CTT	GTAAGTACCA AATGGACTCA		3200
Asp Val Gln Ile Val Val Leu 430			
TTGGTATCTCT CTTGTTGAGT TTACTTGTGC CGAAACTGAA ATTGACCTGC TACTCATCCT ATGCATCAG GGA ACT GGC AAA AAG GAG TTT GAG Gly Thr Gly Lys Lys Glu Phe Glu 435	440		3250 3293
CAG GAG ATT GAA CAG CTC GAA GTG TTG TAC CCT AAC AAA GCT Gln Glu Ile Glu Gln Leu Glu Val Leu Tyr Pro Asn Lys Ala 445	450		3335
AAA GGA GTG GCA AAA TTC AAT GTC CCT TTG GCT CAC ATG ATC Lys Gly Val Ala Lys Phe Asn Val Pro Leu Ala His Met Ile 455	460	465	3377
ACT GCT GGT GCT GAT TTT ATG TTG GTT CCA AGC AGA TTT GAA Thr Ala Gly Ala Asp Phe Met Leu Val Pro Ser Arg Phe Glu 470	475	480	3419
CCT TGT GGT CTC ATT CAG TTA CAT GCT ATG CGA TAT GGA ACA Pro Cys Gly Leu Ile Gln Leu His Ala Met Arg Tyr Gly Thr 485	490	495	3461
GTAAGAACCA GAAGAGCTTG TACCTTTTA CTGAGTTTTT AAAAAAAGAA TCATAAGACC TTGTTTCCA TCTAAAGTTT AATAACCAAC TAAATGTTAC TGCAGCAAGC TTTTCATTTC TGAAAATTGG TTATCTGATT TAAACGTAAT			3511 3561 3611

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CACATGTGAG TCAG GTA CCA ATC TGT GCA TCG ACT GGT GGA CTT	3655
Val Pro Ile Cys Ala Ser Thr Gly Gly Leu	
500 505	
GTT GAC ACT GTG AAA GAA GGC TAT ACT GGA TTC CAT ATG GGA	3697
Val Asp Thr Val Lys Glu Gly Tyr Thr Gly Phe His Met Gly	
510 515 520	
GCC TTC AAT GTT GAA GTATGTGATT TTACATCAAT TGTGTACTTG	3742
Ala Phe Asn Val Glu	
525	
TACATGGTCC ATTCTCGTCT TGATATACCC CTTGTTGCAT AAACATTAAC	3792
TTATTGCTTC TTGAATTGG TTAG TGC GAT GTT GTC GCA GCT	3837
Cys Asp Val Val Asp Pro Ala	
530	
GAT GTG CTT AAG ATA GTA ACA ACA GTT GCT AGA GCT CTT GCA	3879
Asp Val Leu Lys Ile Val Thr Thr Val Ala Arg Ala Leu Ala	
535 540	
GTC TAT GGC ACC CTC GCA TTT GCT GAG ATG ATA AAA AAT TGC	3921
Val Tyr Gly Thr Leu Ala Phe Ala Glu Met Ile Lys Asn Cys	
550 555 560	
ATG TCA GAG GAG CTC TCC TGG AAG GTAAAGTGTGA ATTTGATAAT	3965
Met Ser Glu Glu Leu Ser Trp Lys	
565	
TTGCGTAGGT ACTTCAGTTT GTTGTCTCG TCAGCACTGA TGGATTCCAA	4015
CTGGTGTCT TGCAG GAA CCT GCC AAG AAA TGG GAG ACA TTG	4057
Glu Pro Ala Lys Lys Trp Glu Thr Leu	
570 575	
CTA TTG GGC TTA GGA GCT TCT GGC AGT GAA CCC GGT GTT GAA	4099
Leu Leu Gly Leu Gly Ala Ser Gly Ser Glu Pro Gly Val Glu	
580 585 590	
GGG GAA GAA ATC GCT CCA CTT GCC AAG GAA AAT GTA GCC ACT	4141
Gly Glu Glu Ile Ala Pro Leu Ala Lys Glu Asn Val Ala Thr	
595 600 605	
CCT TAA ATGAGCTTTG GTTATCCTTG TTTCAACAAAT AAGATCATT	4187
Pro ***	
606	
AGCAAACGTA TTTACTAGCG AACTATGTAG AACCTATTA TGGGGTCTCA	4237
ATCATCTACA AAATGATTGG TTTTGCTGG GGAGCAGCAG CATATAAGGC	4287
TGTAAATCC TGGTTAATGT TTTGTAGGT AAGGGCTATT TAAGGTGGTG	4337
TGGATCAAAG TCAATAGAAA ATAGTTATTA CTAACGTTTG CAACTAAATA	4387
CTTACTATG TAGCATAAAT AATACTAGAA CTAGTAGCTA ATATATATGC	4437
GTGAAATTGT TGTACCTTT CTGCAATAAT TATTTGCAGT ACATATATAA	4487
TGAAATTAAC CCAAGGAAATC AATGTTTCTT GCTCCGTCCT CTTTGATGA	4537
TTTTTACCC ATACAGAGC TATGTTTAA TGTATATAAT TTTGTTTAA	4587

AGAAGTAATC	AAATTCAAAT	TAGTTGTTG	GTCATATGAA	AGAAGCTGCC	4637
AGGCTAACTT	TGAGGGAGATG	GCTATTGAAT	TTCAAAATGA	TTATGTGAAA	4687
ACAATGCAAC	ATCTATGTCA	ATCAACACTT	AAATTATTGC	ATTTAGAAAG	4737
ATATTTTGAA	GCCCCATGACA	CATTCAATTCA	AAAAGTAAGG	TAGTATGTAT	4787
GATTGAATGG	ACTACAGCTC	AATCAAAGCA	TCTCCTTTAC	ATAACGGCAC	4837
TGTCCTTGT	CTACTACTCT	ATTGGTAGTA	GTAGTAGTAA	TTTTACAATC	4887
CAAATTGAAT	AGTAATAAGA	TGCTCTCTAT	TTACTAAAGT	AGTAGTATTA	4937
TTCTTCGTT	ACTCTAAAGC	AACAAAA			4964

CLAIMS

1. Method of suppressing amylose formation in potato, characterised by genetically engineered modification of the potato by introducing into the genome of the potato tissue a gene construct comprising a fragment of the potato gene which codes for formation of granule-bound starch synthase (GBSS gene) inserted in the antisense direction, said fragment being selected among the fragments which essentially have the nucleotide sequences stated in SEQ ID No. 1, SEQ ID No. 2 and SEQ ID No. 3 together with a promoter selected among CaMV 35S, patatin I and the GBSS promoter.
- 15 2. Amylopectin-type native starch, characterised in that it has been obtained from potato which has been modified in a genetically engineered manner for suppressing formation of amylose-type starch.
- 20 3. Derivatised amylopectin-type starch, characterised in that it is amylopectin-type starch extracted from potato which has been modified in a genetically engineered manner for suppressing formation of amylose-type starch, said amylopectin-type starch subsequently being derivatised in a chemical, physical or enzymatic manner.
- 25 4. Fragment of the gene coding for granule-bound starch synthase (GBSS) in potato, said fragment being selected among the fragments which essentially have the nucleotide sequences stated in SEQ ID No. 1, SEQ ID No. 2 and SEQ ID No. 3.
- 30 5. Promoter for the gene for granule-bound starch synthase (GBSS) in potato, said promoter being tuber-specific and having essentially the nucleotide sequence stated in SEQ ID No. 4.
- 35 6. Gene coding for granule-bound starch synthase in potato (GBSS gene) having essentially the nucleotide sequence stated in SEQ ID No. 5.

7. Antisense construct for inhibiting expression of the gene for granule-bound starch synthase in potato, comprising

- a promoter,
- a fragment of the gene coding for granule-bound starch synthase inserted in the antisense direction, said fragment being selected among the fragments having essentially the nucleotide sequences stated in SEQ ID No. 1, SEQ ID No. 2 and SEQ ID No. 3.

10 8. Antisense construct as claimed in claim 7, characterised in that the promoter essentially has the sequence stated in SEQ ID No. 4.

9. Antisense construct as claimed in claim 7, characterised in that the promoter is selected among the CaMV 35S promoter and the patatin I promoter.

15 10. Vector comprising a fragment of the gene coding for granule-bound starch synthase (GBSS) in potato, said fragment being selected among the fragments having essentially the nucleotide sequences stated in SEQ ID No. 1, SEQ ID No. 2 and SEQ ID No. 3, and inserted in the antisense direction.

11. Vector comprising the antisense construct as claimed in any one of claims 7-9.

12. Cell of potato plant whose genome comprises the antisense construct as claimed in any one of claims 7-9.

25 13. Potato plant whose genome comprises the antisense construct as claimed in any one of claims 7-9.

14. Potato tubers whose genome comprises the antisense construct as claimed in any one of claims 7-9.

30 15. Seeds from potato plant, whose genome comprises the antisense construct as claimed in any one of claims 7-9.

16. Microtubers of potato, whose genome comprises the antisense construct as claimed in any one of claims 7-9.

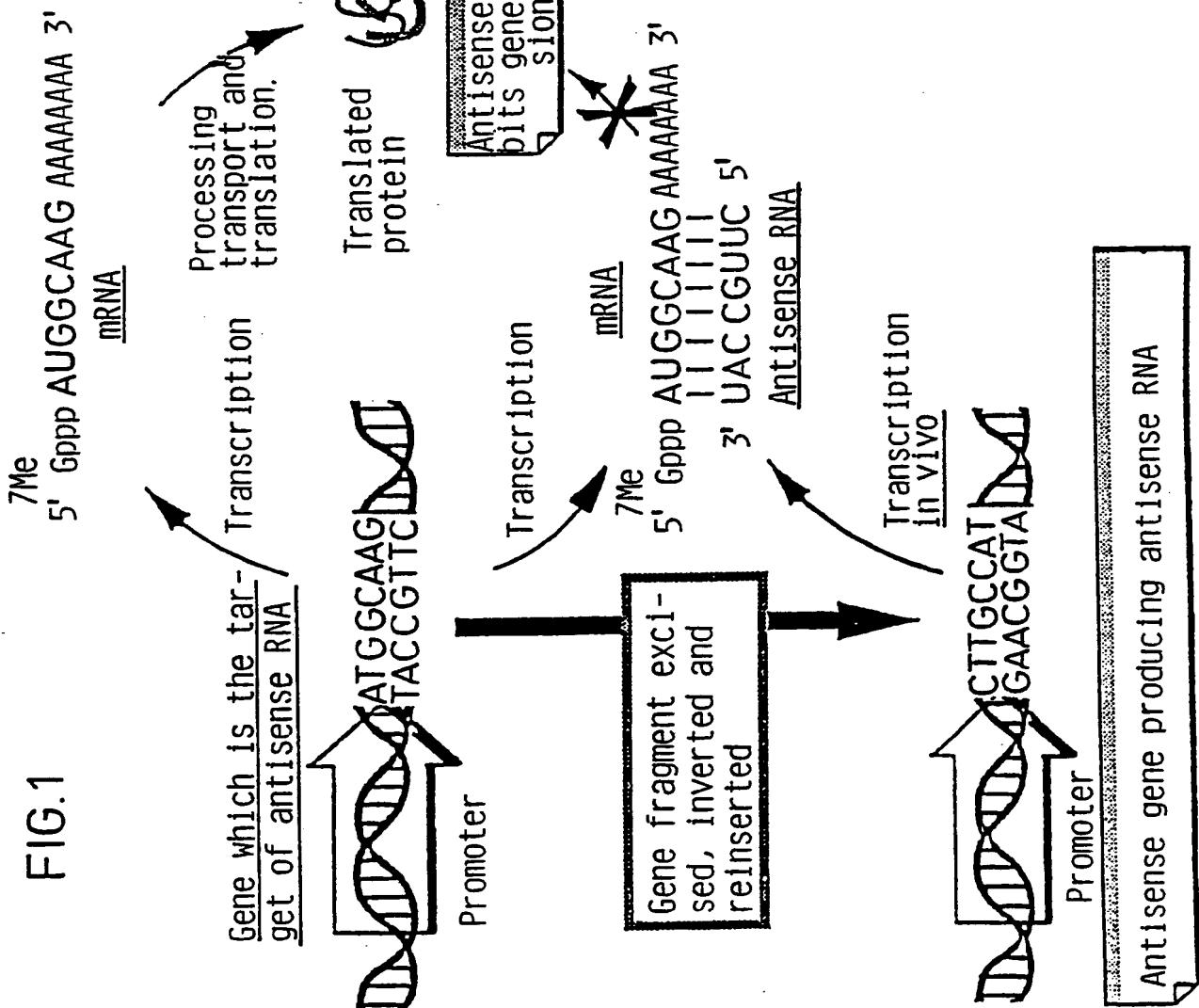


FIG. 2 Result of restriction analysis. GBSS coding region including introns are marked in a darker tone.

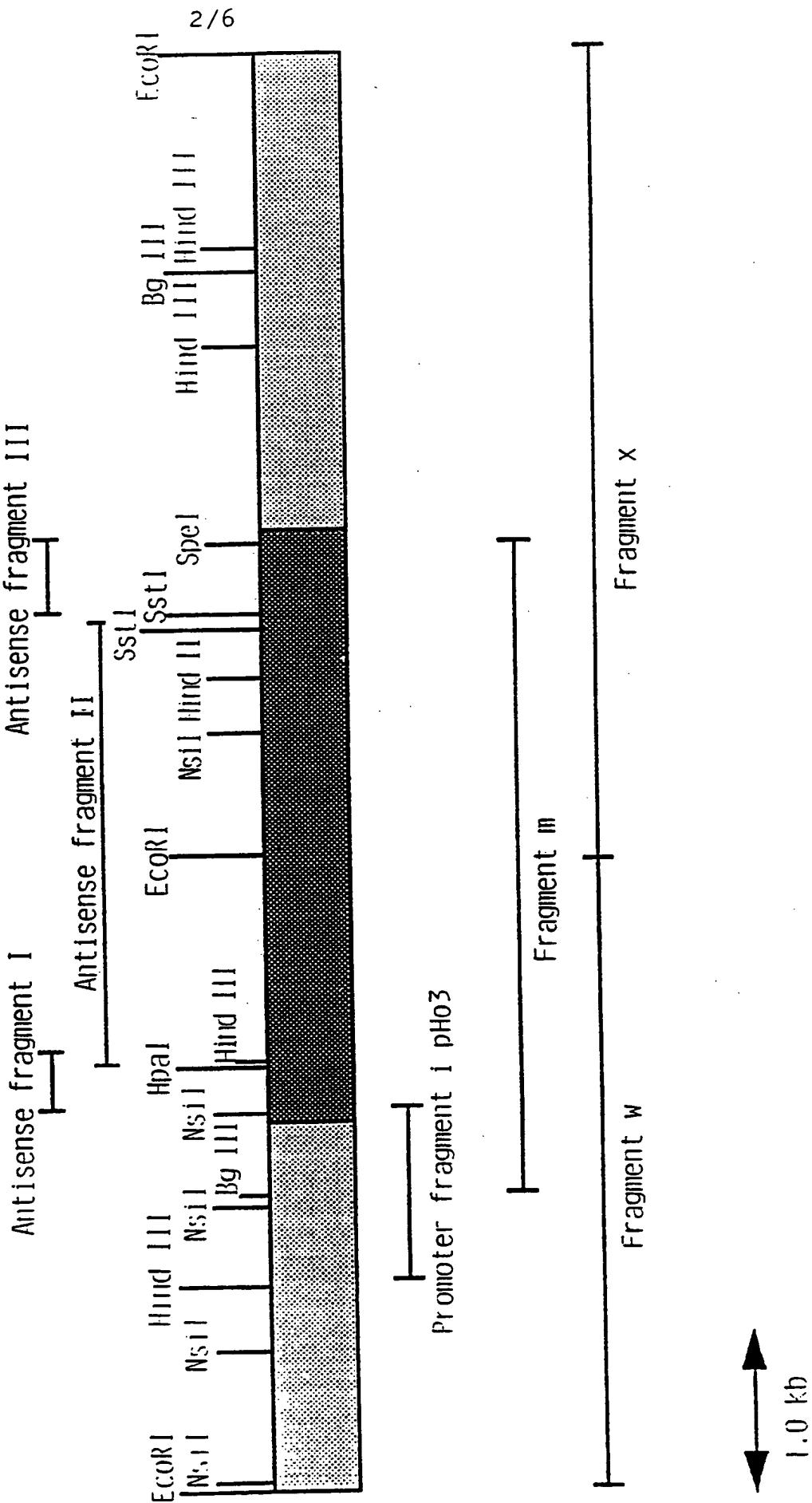


FIG.3

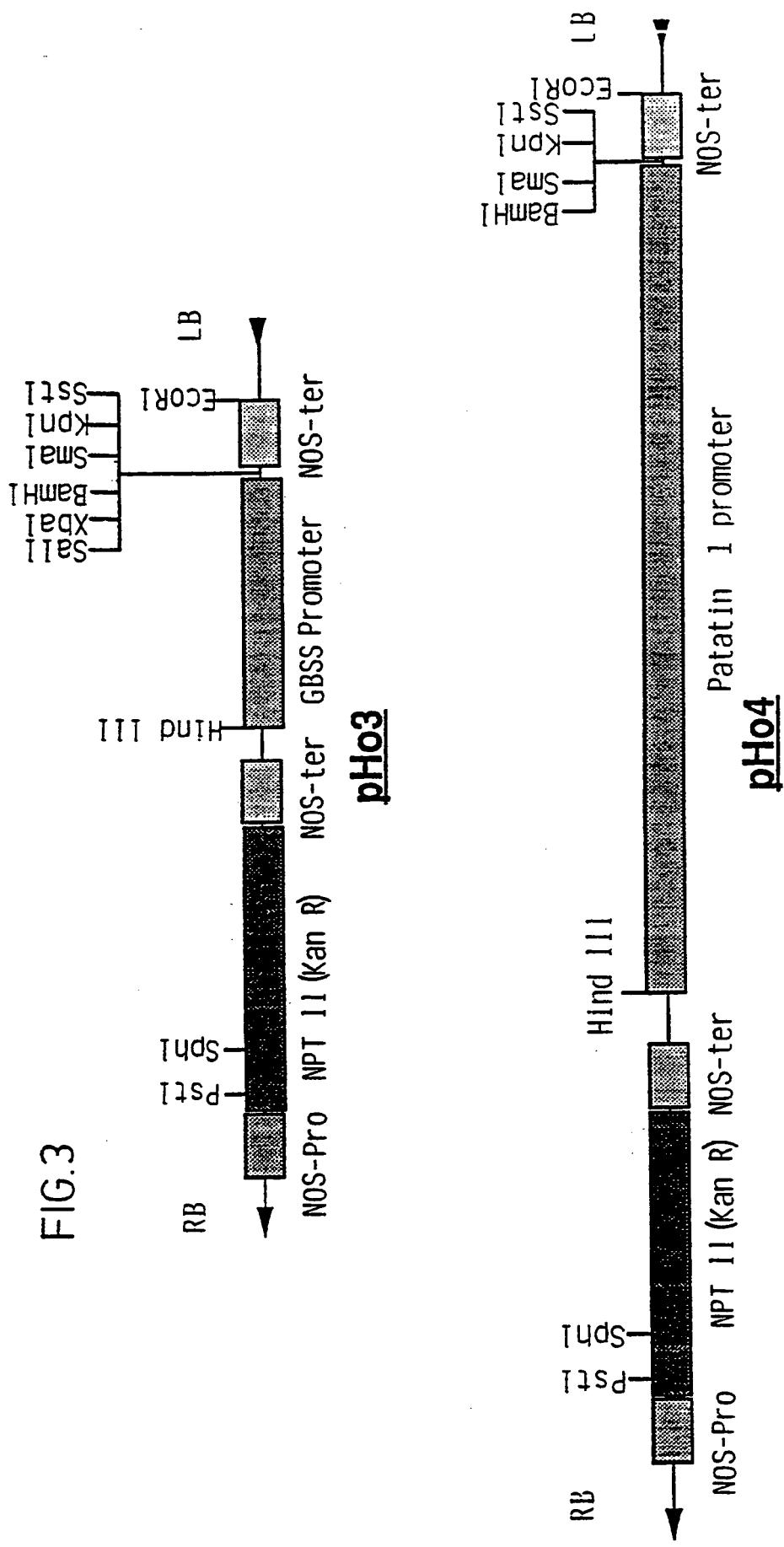


FIG. 4

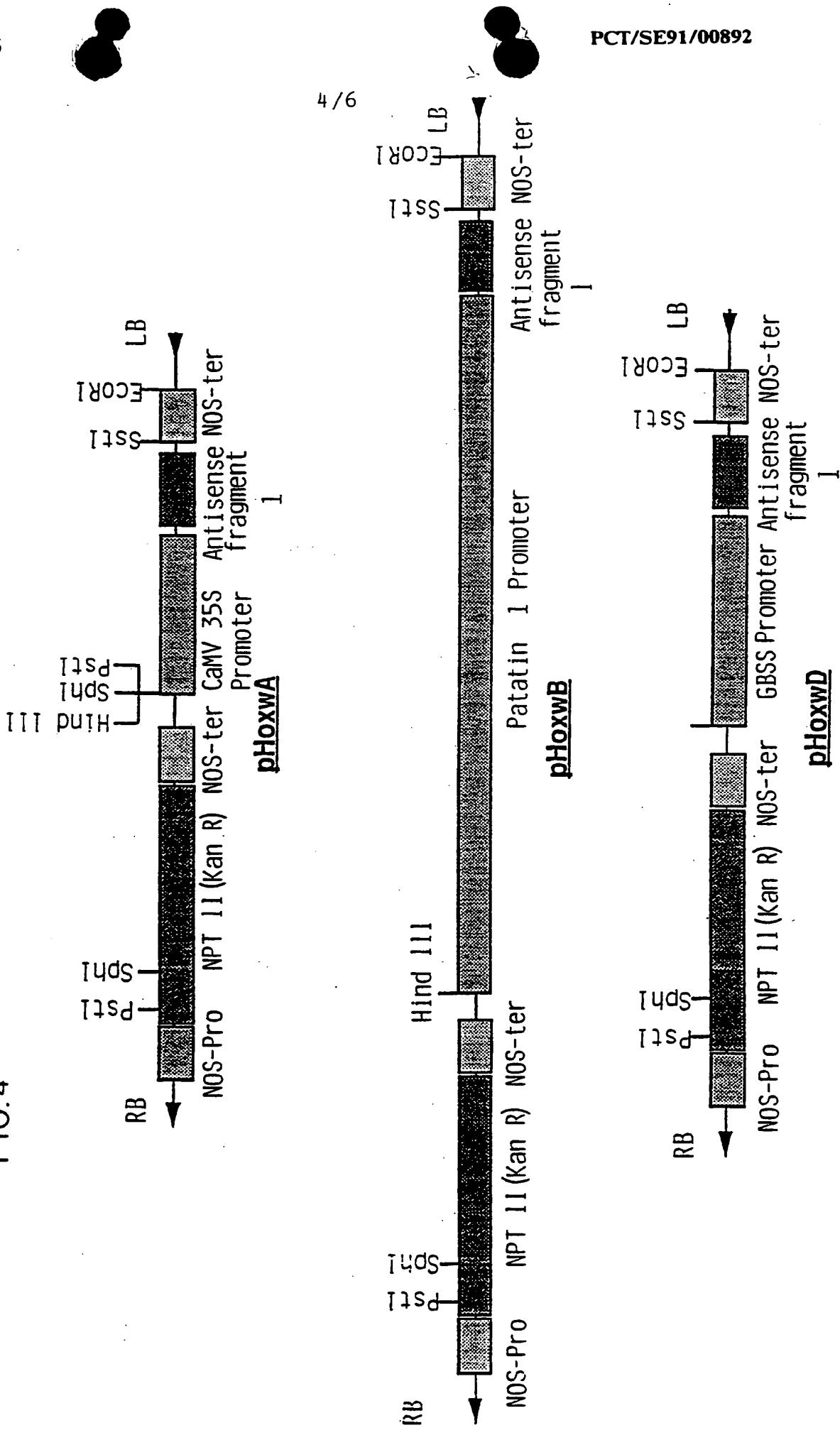


FIG. 5

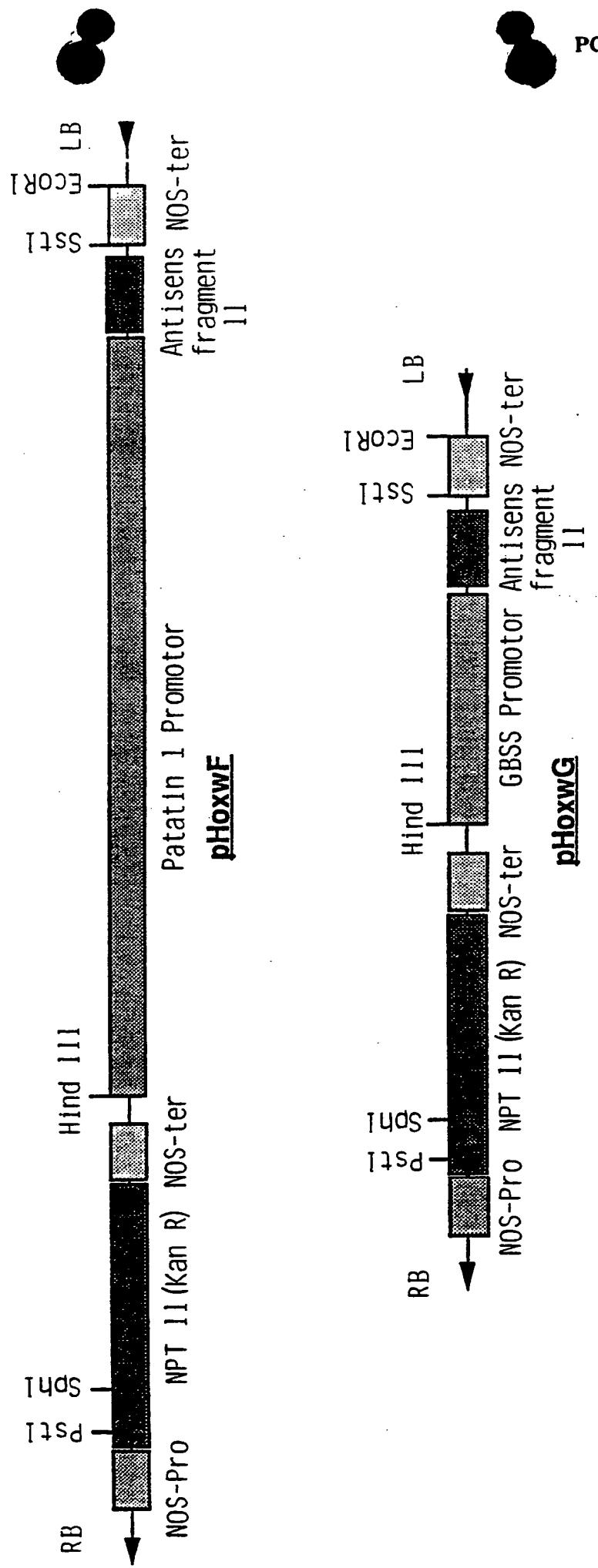
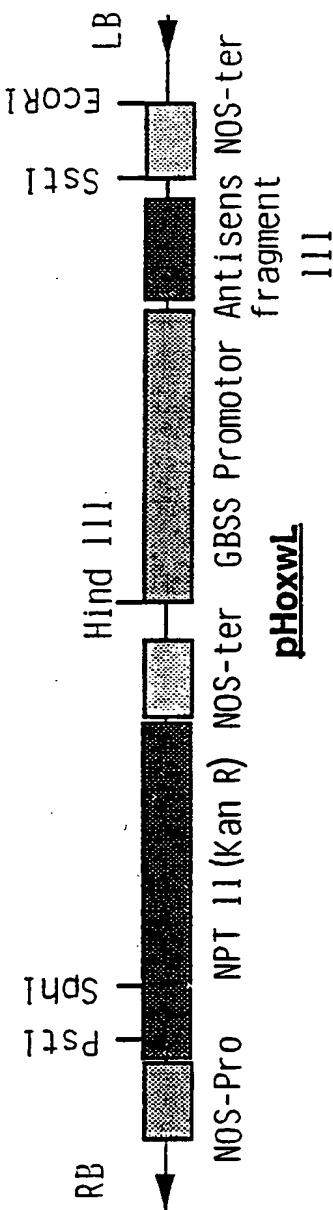
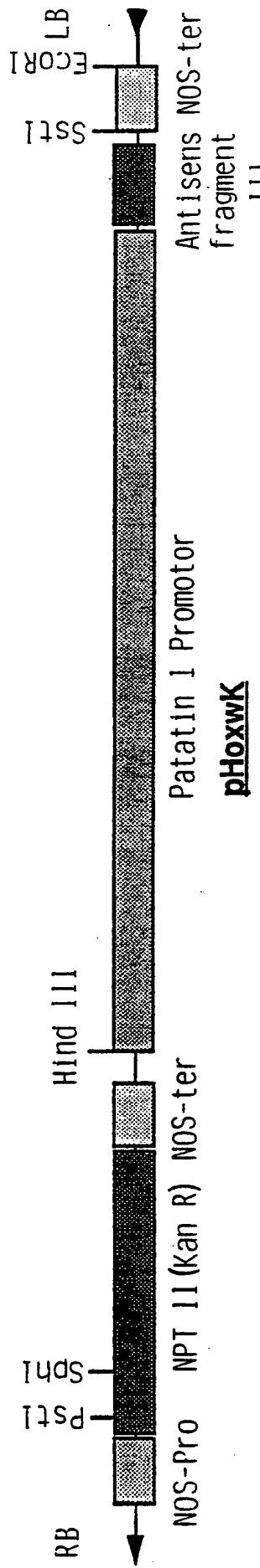


FIG. 6



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INTERNATIONAL SEARCH REPORT

International Application No. PCT/SE 91/00892

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 N 15/56, 9/42, A 01 H 5/00

II. FIELDS SEARCHED

Minimum Documentation Searched⁷

Classification System	Classification Symbols
IPC5	C 12 N; A 01 H

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in Fields Searched⁸

SE, DK, FI, NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
P, X	MOL GEN GENET, Vol. 225, 1991 R.G.F. Visser et al: "Inhibition of the expression of the gene for granule-bound starch synthase in potato by antisense constructs", see page 289 - page 296 ---	1-16
A	EP, A2, 0368506 (IMPERIAL CHEMICAL INDUSTRIES PLC) 16 May 1990, see especially claim 14 ---	1-16
A	PLANT SCIENCE, Vol. 64, 1989 R.G.F. Visser et al: "Molecular cloning and partial characterization of the gene for granule-bound starch synthase from a wildtype and an amylose-free potato(solanum tuberosum L.)", cited in the application ---	1-16

* Special categories of cited documents:¹⁰

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IV. CERTIFICATION

Date of the Actual Completion of the International Search

30th March 1992

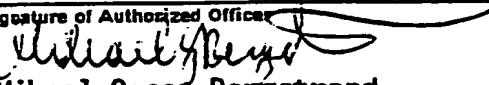
Date of Mailing of this International Search Report

1992-04-01

International Searching Authority

Signature of Authorized Officer

SWEDISH PATENT OFFICE


Mikael G:son Bergstrand

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		Relevant to Claim No
Category	Citation of Document, with indication, where appropriate, of the relevant passages	
A	EP, A2, 0335451 (VERENIGING VOOR CHRISTELIJK WETENSCHAPPELIJK ONDERWIJS) 4 October 1989, see the whole document ----- -----	1-16

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.PCT/SE 91/00892

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 28/02/92.
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A2- 0368506	90-05-16	AU-D- JP-A-	4430789 2273177	90-08-16 90-11-07
EP-A2- 0335451	89-10-04	JP-A- NL-A-	2016985 8800756	90-01-19 89-10-16

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